Original Research

Cytotoxicity Test of 4-Methacryloxyethyl Trimellitic Anhydride–based Dentine Bonding Material Using Acetone Solution in Dental Pulp Fibroblast

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Abstract

Aims and Objectives: Both carious and non-carious lesions covering large dentine areas are indisputable indications of the need to use dentine bonding. Clinically, dental preparation, which is subsequently subjected to dentine bonding application often results in post-restorative pain. Various studies suggest that post-restorative pain is caused by the presence of residual monomers from the imperfect polymerization of a bonding material. The residual monomer can be a free radical that will induce oxidative stress conditions producing a toxic effect on 4-methacryloxyethyl trimellitic anhydride (4-META) monomer as the base material of dentine bonding. The aim of the study was to determine the toxic concentration of 4-META dentine bonding material using acetone as a solvent that destroys 50% of the dental pulp fibroblast cells. **Materials and Methods:** Human pulp fibroblast cells contained in each well were treated with 4-META-acetone solution at concentrations of 5000, 2500, 1250, 625, 312.5, 156.25, 78.12, 39.06, 19.53, and 9.76 µg/mL. Two wells were left untreated to form the control group. A cytotoxicity test was performed by means of an 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-assay test. The optical density of each well was measured with an enzyme-linked immunosorbent assay (ELISA) reader and the percentage of human pulp fibroblast cell destroyed was calculated using the appropriate formula. **Results:** The concentration of 4-META-based dentine bonding with acetone solvent capable of causing 50% human pulp fibroblast cell death (LC_{50}) was 1250 µg/mL. **Conclusion:** Toxic concentrations are those greater than or equal to 1250 µg/mL.

Keywords: Acetone, Cytotoxicity, Dentine Bonding, Fibroblast Cells, 4-Methacryloxyethyl Trimellitic Anhydride

Introduction

Composite resin has positive aesthetic characteristics due to its wide variety of colors and combination of translucence and opacity.[1] However, composite resin restoration of a large lesion involving dentine requires the application of adhesive substances in sufficient enamel that remains. The adhesive materials, referred to as dentin bonding, can adhere to wet dentin surfaces, thus improving the retention of composite resin in composite restoration.[2] The location of carious lesions requiring dentine bonding as an adhesive substance is the deep proximal section, and the surrounding area of a filling requires a strong bond between the dentine and restorative material. Thus, dentine bonding is an absolute necessity in both carious and non-carious lesions involving extensive dentine surfaces.[3] Basically, the working principles of a dentine bonding system are the physical–mechanical bond and the chemical interaction between the dentine bonding material and the collagen tissue located on the subsurface of the dentinal surface.[4] The main purposes of this adhesive system are to create a stable bond between the restorative material and the tooth itself, as well as to cover the dentine surface.[5]

Clinically, tooth preparation for a restorative resin composite using dentin bonding often results in

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post-restorative pain.[6] Various studies suggest that this type of post-restorative pain is caused by the presence of residual monomers from the imperfect polymerization of the bonding material. The unpolymerized monomers may alter the biocompatibility of the material.[5] Previous research confirmed that a large amount of residual monomer was found to be cytotoxic.[5,7] On a certain occasion, the residual monomers can transform into free radicals, which induce an oxidative stress condition.[8] Free radicals represent negative ions that do not bind to other ions in the atomic orbit, thereby attracting other free radicals around them.

There are various commonly used dentine bonding materials, for example, 2-HydroxyEthyl Methacrylate (HEMA), 4-methacryloxyethyl trimellitic anhydride (4-META), Methyl methacrylate (MMA), Triethylene Glycol Dimethacrylate(TEDGMA), among others.[9] Trubiani *et al.*[10] concluded that many of the constituents are likely to cause cellular stress and have a significant cytotoxic effect. Nevertheless, a study by Olivier *et al.*[7] showed that several dentin bondings contain various monomers, dentin bonding containing 4-META being the least toxic. With its low cytotoxicity,[11] hydrophobic and hydrophilic properties, and ability to penetrate the hard tissue of the tooth to form a hybrid layer in dentin,[12] 4-META is a promising material for use as a single monomer in dentin bonding.

In addition to their monomer composition, dentin bonding materials also contain solvent, which plays an important role in diffusing the monomers between collagen fibrils.[13] As a solvent, acetone is commonly used in dentin bonding material due to its ability in attracting the moisture to prevent the breakdown of collagen fibrils before evaporating and being replaced by 4-META, thus resulting in a good binding between monomer and collagen fibrils.[9,13] A study by Prasetyo *et al*.[14] confirmed that 4-META bonding agent featuring acetone solvent shows superior chemical bond strength compared to that containing ethanol solvent.

The cells most affected by dentine bonding are the fibroblast cells contained in the pulp. The level of toxicity can usually be seen through the difference in the number of cells, which undergo apoptosis and necrosis. The toxicity level of a chemical substance is proportionate to its concentration. Usually, the higher the concentration of a substance, the higher the level of toxicity produced. The toxic nature of a chemical substance is determined by LC_{50} (lethal concentration).[15]

Commercial dentine bondings containing 4-META are usually added with many other materials to optimize their effect,[7] which added the cost and made the commercial dentine bonding expensive.

With the aim to improve and widen the treatment to the patient, an inexpensive alternative of dentine bonding agent is needed. Despite the potency of 4-META as a single monomer in dentin bonding agent with acetone as its solvent, which means a lower production cost, a study observing the cytotoxicity of this combination is yet to be conducted. One of the requirements that should be fulfilled by adhesive systems as biomaterials is the biocompatibility and cytotoxicity assessment, which denotes a prerequisite to evaluate the biocompatibility of a material.[16] Thus, this study intended to determine the toxicity concentration of 4-META dissolved in acetone as a bonding agent, which causes 50% mortality of dental pulp fibroblast cells (LC_{50}).

Materials and Methods

This study featured posttest-only control group design. The procedure was approved by the Ethical Clearance Board of the Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia (Certificate no. 122/HRECC.FODM/VIII/2017).

The research samples consisted of dental pulp fibroblast cells cultured from first upper human premolars (P1) extracted for orthodontic treatment purposes. The tooth were collected from the patients after signing a consent form. The inclusion criteria for the tooth were the first upper human premolars (P1) extracted for orthodontic treatment purposes from patients with age range of 16–18 years, with healthy condition without caries or cracks, no tooth mobility, and no periodontal abnormalities. Tooth exclusion criteria were tooth with mobility, dilaceration root, and difficult to extract. These pulp fibroblast cell cultures were prepared 2 months before cell treatment at LPPT Gadjah Mada University, Yogyakarta, Indonesia. The first premolars (P1) of 16-year-olds were washed with phosphate-buffered saline (PBS) before being divided into two sections using a low speed, tungsten carbide round bur no. 014 (MANI, Tochigi, Japan). The pulps were removed, sliced into small pieces with scissors, and subsequently minced using a scalpel. The minced pulps were then placed on petri dishes containing complete media (Dulbecco's Modified Eagle Medium 10%, Fetal Bovine Serum 10%, trypsin 1%, fungizone 0.5 µg/mL, and Pen strep 1%–2%) (Gibco, Welltham, Massachusetts), covered by deck glasses and then placed in the CO_2 incubator to be cultured. The media were changed once every 3–4 days until long, spindle-shaped fibroblast cells appeared [Figure 1].

The procedure of fibroblast cell culture was through some passages until the fibroblast cells were ready to treat. Passage 1: After 80% fibroblast cells grow (seen and calculated under a microscope), the pulp in the petri dish is removed so that it only leaves confluent fibroblast cells. After the cells are confluent, the cells are harvested and

then stripped so that the cells are released from the petri dish wall and then centrifuged. Next the cell is inserted into the flask (the cell is not yet stable). Passage 2: The cells in the flask are replaced by complete media every 3–4 days until 80% fibroblast cells grow (seen and calculated under a microscope). In this phase, cells are developed so that the number is more (subculture). Passage 3: Treatment is the same as passage 2. Cells in passage 3 are considered stable and ready to be treated.

After the three aforementioned passages, fibroblast cells were ready to harvest. There were approximately 80%–90% confluent fibroblast cells (seen under a microscope). Later, dispose the medium in the flask and wash the flask using a medium without FBS. Insert the medium in the flask and then shake to remove the remnants of FBS that are still attached to the cell, and then pour. Add trypsin- ethylenediaminetetraacetic acid (EDTA) 0.25% 1–2 mL and stand for a while, until the cell is released (rounded). Insert into the centrifuge tube, add medium until full. Centrifuge for 10 min at 1500 rpm [Figure 2]. Remove the supernatant and then add 1 mL of complete medium, homogenized. Last, calculate cells obtained (cells ready for treatment).

This study used 4-META gel (Chemlin UK, Nanjing, China), which contains residual solvent (ethyl acetate) and 5% polymerization inhibitor butylhydroxytoluene as the base material of dentine bonding. Pure 4-META gel at a concentration of 95% was then titrated in 100% acetone solution (Cambridge Isotope Laboratories, Cambridge, MA) at specific concentrations (5000, 2500, 1250, 625, 312.5, 156.25, 78.12, 39.06, 19.53, and 9.76 µg/mL).[17]

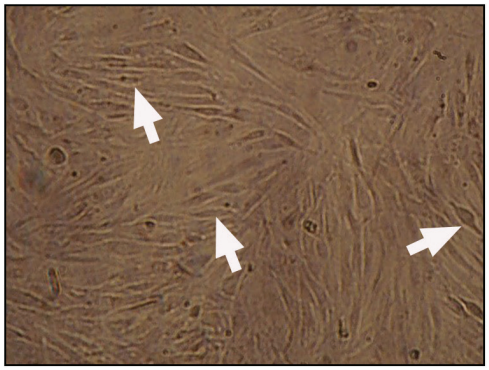

Figure 1: The white arrow shows microscopic images of living fibroblasts

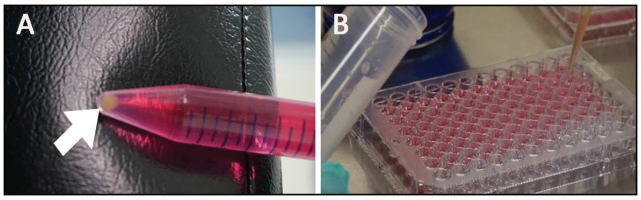

Figure 2: (A) Centrifugation results, white arrow shows the detached cells at the end of the tube. (B) Transfer cells to the 96-well plate as much as 100 µL each

The cells were transferred to a 96-well microplate (IWAKI, Fukushima, Japan), each well containing 100 µL pulp fibroblast cell suspension with a solidity of 2×10^4/20,000 cells/well, before being left for 1–2 h. Three wells were left empty to serve as the media control and three wells were filled with cells as the control. The cells were incubated inside a CO_2 incubator for a minimum of 4 h to enable cell attachment after harvesting (cell sharing). A total of 100 µL of a mixture of 4-META and acetone in specific concentrations were added after incubation to every well of each treatment group (n = 3) using a micropipette, before being incubated inside the CO_2 incubator for 24 h (CO_2 level, 5%, temperature, 37°C, humidity, 98%). After 24 h, the mixture was observed under Digital Carl Zeiss–Axioskop 40 microscope (Carl Zeiss Microscopy, Germany) and photographed by a SONY alpha 5000 camera (SONY, Tokyo, Japan). The wells containing the cells were washed with 100 µL PBS, before 100 µL MTT was added to each one (5 mg 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) + 1 mL PBS + 9 mL complete medium/growing medium). The microplate was then incubated for 4 h until the formation of formazan occurred. Stopper solution of 100 µL Sodium Lauryl Sulfate 10% was added to the 0.01 N HCl in each well and the microplates were incubated overnight. A total of 50 µL of dimethyl sulfoxide was added to each well, before being agitated for 5 min until the formazan crystals were completely diluted. Thereafter, they were counted using an enzyme-linked immunosorbent assay (ELISA) Reader Bio-RAD 680 XR (Bio-RAD Laboratories, Hercules, California) at a wavelength of 550 nm. Blue color indicating the presence of formazan formation was found in the well containing living cells, whereas no color was found in the well devoid of living cells. The optical density recorded was then calculated using the Meyer formula:[18]

$$\%\text{Cell death} = \frac{\text{Control OD - Sample OD}}{\text{Control OD}} \times 100\%$$

Explanation: OD = Optical density

The data obtained was analyzed with the Statistical Package for the Social Sciences (SPSS) software, version 24 (IBM, Armonk, New York) using a Kruskal–Wallis test to identify the differences between all sample groups and a Tukey HSD test to establish the differences between each group. All samples were statistically analyzed with a confidence interval of 95% ($P \leq 0.05$).

Results

On the basis of toxicity test of 4-META-based dentine bonding material using acetone solution in dental pulp fibroblast cells, the percentages of fibroblast cell death calculated are present in Table 1.

As the data were in homogen, the differences among groups were analyzed using Kruskal–Wallis test, which showed a significant difference among treatment groups (P = 0.001; 95% CI). The table shows that the concentration level resulting in 50% fibroblast cell death (LC_{50}) is one of 1250 µg/mL. This means that the toxicity level of 4-META for the base material of dentine bonding in acetone solution is higher or equal to 1250 µg/mL.

On the basis of Tukey HSD, significant differences were shown if only the mean value of each concentration belongs to different subset column. As shown in Table 1, 4-META-based dentine bonding material in acetone at 2500 µg/mL has no significant difference compared to 5000 µg/mL concentration. However, 1250 µg/mL concentration shows a significant difference compared to the other concentration. Thus, the significant difference means that the value of LC_{50} in 4-META-based dentine bonding material in acetone was at a concentration of 1250 µg/mL. An image of microscopic living fibroblast cell, the necrotic fibroblast cell, and the lytic nucleus is shown in Figure 3.

Discussion

Dentine bonding is an agent used as a material to bond the restorative composite with dental tissues. It is often used together with composite resin to decrease the potential for microleakage in filling materials and dental surfaces while also promoting retention of the filling material. Generally, this material is a derivative of methacrylate resin, which is readily polymerized.[5]

To penetrate the dental tissues, a dentine bonding should contain a component called solvent whose function is to help dilute the monomers and facilitate their penetration of demineralized dentine.[19] All materials applied to living tissues have to be biocompatible meaning that the material can be absorbed by the body and does not cause any subsequent abnormalities or pathologic conditions. It also denotes that the material is neither toxic nor dangerous if applied to living tissues.[13] Considering the close contact with dentine and vital pulp tissue, also in case of accidental contact with the other tissue in the oral cavity, a cytotoxicity test of the dentin adhesive material is necessary. However, cytotoxicity assessment of a material is mostly performed in cell culture due to ethical reasons.[16]

This research is based on several clinical phenomena related to postoperative pain after filling with composite resin and preceding the application of dentine bonding.[20] Several research mention that the free monomers of dentine bonding materials are suspected of being one cause of postoperative pain and of teeth getting

Table 1: Percentages of fibroblast cell death in 10 concentrations of 4-META in different acetone solutions

No.	Concentration of 4-META in acetone solution	Average of optical density	% Cell death	*P* value
1	5000 µg/mL	0.091[a]	88.70%	0.001
2	2500 µg/mL	0.084[a]	89.60%	
3	1250 µg/mL	0.402[b]	50.30%	
4	625 µg/mL	0.555[c]	31.40%	
5	312,5 µg/mL	0.626[d,e]	22.60%	
6	156,25 µg/mL	0.632[d,e]	21.80%	
7	78,12 µg/mL	0.654[e]	19.16%	
8	39,06 µg/mL	0.592[c,d]	26.80%	
9	19,53 µg/mL	0.598[c,d]	27.10%	
10	9,76 µg/mL	0.670[e]	17.20%	
11	Control	0.68[f]	0%	

[a,b,c,d,e,f]Different superscript denotes significant difference ($P \leq 0.05$)

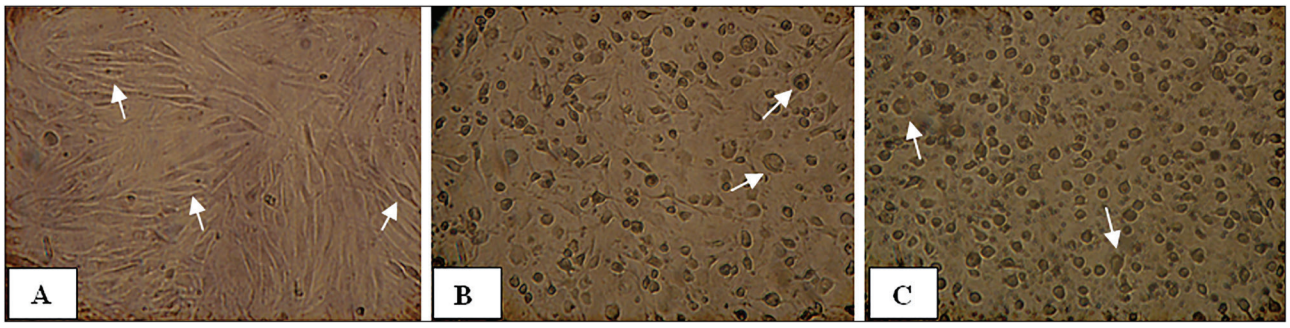

Figure 3: (A) Microscopic picture of living fibroblast cell (white arrows). (B) Microscopic picture of necrotic fibroblast cell, round, bulging with nucleus fragmentation (white arrows). (C) Lytic nucleus cannot be seen therefore the empty cavities bordered by nucleus membrane are called ghosts (white arrows)

progressively necrotized.[21] Pupo *et al.*[22] analyzed the metabolic effects of several adhesive materials and revealed that the toxicity of the current adhesive systems varied, they suspected that it was likely related to the level of residual monomers. These free monomers have also been considered capable of damaging fibroblast cells in dentine and pulp tissue.[5]

One of the most basic ingredients of the dentine bonding used is 4-META. Several studies have revealed that 4-META monomers can induce the death of surrounding tissues by producing free radicals from residual monomers because of imperfect polymerization.[21]

During the hydrolysis process, 4-META monomer contains a number of groups (-OH), which are forms of free radical reactive compounds called reactive oxygen species.[3] In mitochondria, 4-META undergoes hydrolysis to become 4-MET whose chemical structure contains two hydroxyl groups (-OH). This allows 4-META to induce a toxic effect on the surrounding tissues causing cell death. In addition, high free monomer concentrations in registered cells can cause immunosuppression.[5]

There are several biochemical mechanisms thought to mediate cell death, which explain its occurrence because of the toxicity of a substance. Free radicals in cells cause oxidative stresses, which will result in the hyperactivation of poly (Adenosine Diphosphate (ADP)-ribose) polymerase-1 (PARP-1).[23] PARP-1 hyperactivation will, in turn, inhibit glycolysis and ATP production. The inhibition of ATP production reduces the mount of Adenosine Triphosphate (ATP) and increases the free phosphate levels (the pH in cells increases). ATP constitutes a form of energy required by cells for various functional cell activities. Reduced ATP will disrupt cell activity, culminating in apoptosis.[24] On the contrary, to manage reduced ATP production, cells carry out anaerobic glycolysis using residual glycogen and glucose reserves from surrounding tissue fluid. This leads to the accumulation of lactic acid and lower intracellular pH.[25]

When the cell tries to neutralize the pH, there is an interference with the transition of Na^+ and Ca^{2+} ions to the mitochondria. The ion exchange process requires ATP.[26] When the amount of ATP decreases, interference to this process results, which ultimately causes the Ca^{2+} in the mitochondria to increase to excessive levels.[27] Oxidative stress and pH recovery become normal combined with the overabundant presence of calcium in the cell and other factors such as high phosphate concentrations, causing the mitochondria to experience permeability transition. The opening of the mitochondrial permeability transition pore (MPTP) causes mitochondrial permeability transition (MPT), followed by the occurrence of mitochondrial internal membrane depolarization, matrix swelling, and rupture of the mitochondrial outer membrane.[25,28]

Morphological change constitutes a cell response to stress. Cell stress can be caused by substances that are toxic, mutagenic, and oxidative.[29] Living fibroblasts are spindle-shaped, branching, and elongated with slim branches.[30]

According to Torabinejad *et al.*,[31] fibroblast cells are large, stellate-formed, resemble stars, and possess branches. If affected by lesions, they will experience morphological change characterized by the round-shaped cell, swollen because of nucleus fragmentation. The lytic nucleus cannot be seen, resulting in the empty cavities bordered by nucleus membrane being referred to as ghosts. This type of cell is a form of necrotizing fibroblast cell.[25]

In addition to the presence of residual monomers, that of acetone solvents also influences the presence of free radicals produced by 4-META monomers. Acetone is known to be a volatile solvent with weak hydrogen-binding capacity resulting in the poor performance of the antioxidant mechanism involved in free radical binding.[13]

According to Bakir *et al.*,[5] 4-META monomers induce adverse effects including cell death in tissues. The clinical procedure described earlier supports the aim of this study in determining the minimum toxic concentration of 4-META-based dentine bonding material in acetone solution, which can cause the death of 50% of dental pulp fibroblasts. By establishing the minimum toxic concentration of this dentine bonding material, dentists and researchers can understand more fully the safe concentrations, which should be used as dentin bonding. Moreover, this research is expected to give a description of the possibility in developing a new dentine bonding material with minimal toxicity.

The data show a significant difference among groups, which means that the concentration of 4-META in the dentine bonding in acetone solution contributes to the cell deaths. The bigger the concentration, the more toxic the bonding material. In the table containing the percentage of fibroblast cell deaths, it can be seen that not all decreases in the concentration of 4-META-based dentine bonding material using acetone solution result in a reduction in the percentage of fibroblast cell death. Certain lower concentrations were found to cause an increase in the percentage of fibroblast cell death. The Tukey HSD test results also confirmed insignificant levels of fibroblast cell death percentage decrease, even though the concentration decrease was significant. This was probably caused by acetone evaporation that increases 4-META concentration.[29] The toxicity test was conducted on 4-META-based dentine bonding material in acetone solution to identify the toxic concentration, which causes 50% mortality among fibroblast cells. The results of this study show that a concentration of 1250 µg/mL kills 50.30% of dental pulp fibroblast cells. Thus, the LC_{50} of this 4-META-based dentine bonding material is 1250 µg/mL.

The limitation of this study was the evaporation of acetone solvent was hard to control, resulting in a really short duration of application, which will give a difficulty in handling the bonding material. Thus, in the future, further improvement in its physical characteristic is needed.

Study results concluded that the concentration of 4-META-based dentine bonding material in acetone solution higher or equal to 1250 µg/mL is toxic, whereas a concentration lower than 1250 µg/mL is safe for dental pulp fibroblast cells.

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Conflicts of interest

There are no conflicts of interest.

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